RAT CONNECTIVE TISSUE RESPONSE TO IMPLANT OF HUMAN DENTAL ROOT TREATED WITH CITRIC ACID AND HUMAN RECOMBINANT PLATELET-DERIVED GROWTH FACTOR-BB (rhPDGF-BB)*

RESPOSTA DO TECIDO CONJUNTIVO DO RATO AO IMPLANTE DE RAIZ DE DENTE HUMANO TRATADO COM ÁCIDO CÍTRICO E FATOR DE CRESCIMENTO DERIVADO DE PLAQUETAS RECOMBINANTE HUMANO (rhPDGF-BB)

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*Resumo da Dissertação apresentada à Faculdade de Odontologia de Bauru - USP, como parte dos requisitos para a obtenção do título de Mestre em Odontologia, área de Periodontia.

This study assessed the connective tissue response to periodontitis-affected root surface after treatment with PDGF-BB, comparing this therapy with root planning and surface demineralization with citric acid. After surface therapy all the root specimens were implanted into incisional wounds on the dorsal region of the rats and collected 3, 7 and 15 days after implantation. Histologic and morphometric analysis included counts of cell densities and evaluation of the repairing tissue adjacent to specimens. It was concluded that root specimen pathogenicity was reduced by citric acid demineralization. It seems that PDGF-BB stimulated connective tissue repair and fiber deposition during the first week.

Key words: Growth factor, platelet-derived; Surface demineralization; Wound healing; Root conditioning; Periodontal disease, regeneration.

INTRODUCTION

The rationale for many of the periodontal therapies is regeneration of the periodontium, composed of alveolar bone, ligament and root cementum. However, most of the time these treatments can lead to only a partial regeneration, a repair, the healing process that does not lead to the complete replacement of the lost tissues[2], rather than regeneration results[4]. This knowledge has focused attention on the more basic aspects of wound healing in the hope of identifying and controling important variables for periodontal regeneration, which include root surface treatment and growth factor enrichment.

Polypeptide growth factors are a class of natural ocurring proteins that mediate key cellular events in tissue repair including cell proliferation, chemotaxis, differentiation and matrix synthesis via binding to cell-surface receptors[2]. Recent research has revealed a number of growth factors that potentialy could be used therapeutically, including the platelet-derived growth factor (PDGF). PDGF have profound effects on chemotaxis and proliferation for periodontal ligament fibroblasts [18,7,23] and bone cells[10, 13]. Animal studies by several investigational groups have demonstrated that PDGF-BB, alone[5,24,9] or combined with other substances[9, 32, 33, 16], has the capacity to stimulate regeneration with new bone, periodontal ligament, and cementum formation in periodontal lesions.

Surface demineralization and application of PDGF-BB on root surfaces have been described for the treatment of periodontal lesions in dogs [5, 24] and for in vitro assessment of fibroblasts cultures[8]. Since this procedure has shown positive results, the present study was undertaken to evaluate the connective tissue response to PDGF-BB treatment of periodontitis-affected root surfaces, as a complementary procedure for root planning and surface demineralization.

MATERIAL AND METHODS

The method for evaluating connective tissue response in vivo used an implantation animal model system [20, 35]. Root specimens for implantation were prepared from periodontitis-affected human root surfaces covered by calculus. In order to prepare the implant specimens, four grooves were disked through the calculus creating a rectangular area. Then the calculus within the rectangular area was removed by curetting for a total of 20 strokes with 5/6 Gracey curette. The methods of preparation and storage used were previously described[29]. Each implant specimen was rectangular in shape, with dimensions of approximately 3 mm x 1 mm x 1mm. All the analysis surfaces had been covered by calculus and exposed to a periodontal pocket - the pulpal dentin surface had been notched to distinguish it clinically and histologically from the opposite surface.

Experimental procedures

The experimental animals consisted of Wistar rats, 8 weeks old, weighing aproximately 160g, and which were maintained on an ad libtum diet of food. The animals were sedated for experimental procedures with ethyl ether. The specimens were implanted subcutaneously into incisional wounds made in the back of the rat's neck. Prior implantation, tricotomy and antisepsis with alcohool 70% of the area were performed. Three incisions were made with the point of a scalpel blade. A large hole point scisor was inserted into the incisions to create a pathway for the specimen over the animal muscle tissue.

Prior to implantation, specimens were treated according to the study group:

Group 1: specimens were flooded with sterile saline;

Group 2: specimens were etched with cotton pellets soaked in an aqueous solution of citric acid* for 3 minutes. Following etching, the roots were profusely flooded with sterile saline;

Group 3: specimens received the same treatment as that of group 2, and 20ml rhPDGF-BB solution in a concentration of 100mg/ml** were applied to root surface using a micropipette***.

Immediately after treatment, one specimen of each group was implanted and wounds were sutured with silk suture.

Animals were sacrificed with ether overdose at 3, 7 and 15 days following placement of implants. Four experimental and four control specimens were available at each time point. Immediately after sacrifice, the area of skin and underling connective tissue containing the specimens were biopsied and processed for histological sectioning: specimens were fixed in 10% phosphate buffered formaline pH7.2 for 72 hours; decalcified in Morse solution for 45 days, embedding in paraffin plusplastic resin****, and sectioned in a plane which resulted in sections exhibiting both the root surface and the notched pulpal surface dentin. Semi-serial sections of 5mm separeted intervals of 50 mm were stained with hematoxylin and eosin.

Analysis Methods

A histologic and histometric analyses were done using 5 sections previously chosen at random. The level of tissue maturity and organization was assessed histologically. The morphometric analysis was done to 7

* pH 1, prepared in the Bauru School of Dentistry Department of Biochemistry, University of São Paulo
** Genzyme Co., Cambridge, MA
*** Pipetman®, Gilson Medical Eletronics (France) S. A.
**** Paraplast, Kerr, USA.

days and 15 days specimens, under a magnification of 1000X, with a Zeiss Kpl 8X eyepiece containing a Zeiss II an integrating grid, positioned over 4 microscopical fields systematically casualized in each section. The total of 20 fields for each specimen was considered representative of the whole for morphometric analysis when a sample homogeneity test was performed according to previous indications[20]. In order to evaluate the numerical density of fibroblast-like cells and inflammatory cells, the Aherne II morphometric method was used[1].

Data Management

The numerical density of cell was used to make a comparison between groups at each 7 and 15 days, and within groups across the different time points. Statistical analysis was made using a two-way analysis of variance. In the event of a significant difference, the Student-Newman Keuls multiple comparison test was used. A level of $P<0.05$ was accepted for statistical significance.

RESULTS

Histologic Analysis

The histologic analysis considered specimens collected from the same rate for comparison between groups in order to eliminate the influence of natural variations between animals.

3 days **.** Artifactual separation of the adjacent connective tissue from the implant was a frequent observation, and the separation tended to leave minimal amounts of material on the implant surface. This artifact was more frequent for groups 1 and 2.

An overview of group 1 specimens showed lots of red blood cells and predominantly polymorphonuclear inflammatory infiltrate. Cells were enmeshed within a delicate fibrin network. These caracteristics were suggestive of coagulum.

In group 2 specimens the majority of cells appeared to be red blood cells and polymorphonuclear inflammatory cells. Some fibrin and mononuclear cells could be distinguished.

Group 3 specimens showed predominantly fibroblast-like cells and mononuclear inflammatory cells. Some polymorphonuclear cells could be observed. Cell attachment to the implant surface was evident , and cells were surrounded by an intercellular matrix, containing some fibers, which had an intimate relationship with the implant surface.

7 days. Fibrin and red blood cells in a loose arrangement were present in group 1 specimens, but less frequently than at 3 days. Connective tissue was rich in blood vessels containing inflammatory cells. Most of cells appeared to be fibroblasts and inflammatory cells, and they appeared not to attach to the implant surface. Inflammatory cells density was significantly higher than in the other groups (Table 1).

Contrary to group 1, cells and fibers in groups 2 and 3 were in contact to specimens surface. In group 1 there was some fibrin,which was not frequent. The majority of cells was mononuclear and fibroblast-like cells. The tissue was rich in blood vessels.

Group 3 specimens could be easily distinguished from groups 1 and 2 because of the apparent high density of fibers. The number of cells neighbouring the implants were apparently lower than in the other groups. Most of cells were fibroblast-like and mononuclear cells. Inflammatory cells density was significantly lower than in the other groups (Table 1). Blood vessels were relatively rare finding.

15 days. In group 1 specimens connective tissue was more mature, containing more organized fibers and less blood vessels than at 7 days. The macrophages density significantly lowered (Table 3). However, the inflammatory infiltrate with neutrophils and macrophages, was present among the implants and the connective tissue. Macrophages, neutrophils, lymphocytes and plasma cells densities were significantly

TABLE 1 – Comparation between mean values (mean ± standard error measurement, X 10^4 cells/mm^3) of fibroblasts, neutrophils, macrophages, lymphocytes and plasma cells in groups 1, 2 and 3, after 7 days

	Group 1	Group 2	Group 3	P*
Fibroblast	36.9 ± 11.77	28.7 ± 5.00	27.2 ± 5.09	0.234
Neutrophil	6.4 ± 3.36^{G3}	4.1 ± 1.86^{G3}	1.5 ± 0.48^{G1,G2}	0.005
Macrophage	10.4 ± 1.37^{G2,G3}	7.7 ± 1.58^{G1,G3}	2.9 ± 1.71^{G1,G2}	0.0002
Lym. + Plasm.	1.0 ± 0.49	0.9 ± 0.60	0.7 ± 0.57	0.746

Lym. + Plasm. = lymphocyte and plasma cell; * = obtained through variance analysis; *G1* = different from group 1; *G2* = different from group 2; *G3* = different from group 3.

TABLE 2 – Comparation between mean values (mean ± standard error measurement, X 10^4 cells/mm^3) of fibroblasts, neutrophils, macrophages, lymphocytes and plasma cells in groups 1, 2 and 3, after 15 days

	Group 1	Group 2	Group 3	P*
Fibroblast	40.4 ± 5.51	40.8 ± 8.83	46.2 ± 5.94	0.445
Neutrophil	9.2 ± 4.46^{G2,G3}	2.4 ± 1.39^{G1}	1.5 ± 0.31^{G1}	0.005
Macrophage	4.1 ± 1.20^{G2,G3}	2.4 ± 0.62^{G1,G3}	1.1 ± 0.43^{G1,G2}	0.002
Lym. + Plasm.	1.4 ± 0.12^{G2,G3}	0.6 ± 0.39^{G1,G3}	0.1 ± 0.21^{G1,G2}	0.0003

Lym. + Plasm. = lymphocyte and plasma cell; * = obtained through variance analysis *G1* = different from group 1; *G2* = different from group 2; *G3* = different from group 3.

TABLE 3 – Comparation of the mean values (mean ± standard error measurement, X 10^4 cells/mm^3) of fibroblasts, neutrophils, macrophages, lymphocytes and plasma cells in groups 1, 2 and 3, between 7 and 15 days analysis

	Group 1	P*	Group 2	P*	Group 3	P*
Fibroblast	7d 36.9 ± 11.77	0.603	7d 28.7 ± 5.00	0.054	7d 27.2 ± 5.09	0.003
	15d 40.4 ± 5.51		15d 40.8 ± 8.83		15d 46.2 ± 5.94	
Neutrophil	7d 6.4 ± 3.36	0.347	7d 4.1 ± 1.86	0.193	7d 1.5 ± 0.48	0.959
	15d 9.2 ± 4.46		15d 2.4 ± 1.39		15d 1.5 ± 0.31	
Macrophage	7d 10.4 ± 1.37	0.001	7d 7.7 ± 1.58	0.001	7d 2.9 ± 1.71	0.087
	15d 4.1 ± 1.20		15d 2.4 ± 0.62		15d 1.1 ± 0.43	
Lym + Plasm	7d 1.0 ± 0.49	0.343	7d 0.9 ± 0.60	0.053	7d 0.7 ± 0.57	0.114
	15d 1.4 ± 0.12		15d 0.6 ± 0.39		15d 0.1 ± 0.21	

Lym. + Plasm. = lymphocyte and plasma cell; * = obtained through variance analysis *G1* = different from group 1; *G2* = different from group 2; *G3* = different from group 3.

higher than in the other groups (Table 2).

Group 2 specimens had less blood vessels than at 7 days, but some restricted areas appeared inflammed. Fibroblast-like cells neighboring the implants had contact with the surfaces. Neutrophils, macrophages, lymphocytes and plasma cells densities were significantly lower than in group1 specimens (Table 2). Macrophages density lowered significantly comparing to 7 days observation (Table 3).

Connective tissue containing predominantly fibroblast-like cells was well attached to group 3 specimens surface. At this time point the group 2 and group 3 specimens could not be well distinguished in a subjective analysis. On the other hand, the morphometric analysis showed macrophages, lymphocytes and plasma cells densities in group 3 statistically lower than in group 2.

Most of implant surface fragments consisting of dentin was a general observation, and root resorption areas were apparently rare at all time points.

DISCUSSION

Root surface treatment, with root planning, demineralization and PDGF-BB application, accelerates regeneration of periodontal defects in dogs but the ankylosis incidence is high[5]. On the other hand, PDGF-BB promotes fibroblasts ligament cells proliferation, chemotaxis and adhesion to root surface[20]. The same therapy, associated to guided tissue regeneration with membranes, enhances regeneration compared to membranes alone, preventing ankylosis[5, 24].

This study focuses on the connective tissue response to periodontitis-affected root surfaces treated with root planning, citric acid conditioning and PDGF-BB application. Other authors used similar experimental procedures to evaluate connective tissue response to citric acid conditioning[28, 33] and root planning[31] and showed that acid conditioning promotes cells and fibers adhesion to dentin and to non-periodontitis-affected cementum, as well as, 20 strokes with Gracey curettes make periodontitis-affected root surfaces biologically acceptable. Based on these works, the present study compared root planning alone (group 1) to root planning followed by citric acid conditioning (group 2) and to the PDGF-BB treatment as a complementary treatment to these procedures.

Since 3 days of repair process, the treatment with the growth factor seemed to accelerate the process. At this moment group 3 specimens were the only ones characterized by fibroblast-like and macrophage-like cells. At 7 days of repair, group 3 specimens showed the connective tissue rich in collagen fibers adjacent to the root surfaces. Fibers production seemed to be more intense than in the other group specimens. This finding is in accordance with Pierce et al. [25]. These authors, studing wounds repair on rats dorsal area, showed collagenous synthesis at 3 days when the wounds were treated with PDGF-BB. The growth factor also promoted marked increases of procolagenous type I in fibroblasts during the first week.

The mechanism of fibers synthesis induction by PDGF-BB is not clear. According to Pierce et al. [26], although the concentration of fibroblasts were substantially greater in the growth factor-treated groups, the proportion of fibroblasts containing procollagen 3 to 7 days after wounding were similar. PDGF-BB was shown to induce expression of TGF-ß in a monocyte line[27] and though may act through the monocyte/macrophage to provide TGF-ß 1 for stimulation of procollagen synthesis in fibroblasts[26, 27]. As in this work and in the results presented by Pierce et al.[26] macrophages were not prominent in tissue sections of PDGF-BB treated wounds after 7 days, this suggest that PDGF might act at the level of fibroblasts in 7 days

wounds over and above its influence on macrophages.

At 15 days the concentration of fibers could not be distinguished between groups. It suggests that the PDGF-BB stimulation to fibers synthesis was transitory and is in accordance with the previous studies that showed a transitory influence of the factor during wound healing[12, 14]. However, this could not be a negative point to wound healing because different authors[25, 12, 30, 24] and the present study demonstrated wound healing acceleration promoted by PDGF-BB during the first days of process, a critical time to fibroblasts repopulation of root surfaces in periodontal defects.

Histomorphometric analysis showed concentrations of inflammatory cells in group 1 specimens higher than in the other groups specimens. As the root fragments were periodontitis-affected, this result may be due to endotoxins contamination. There are endotoxin contaminants on root surfaces after manual or ultra-sonic root planning[22]. Vial bacteries are present in the root cementum and dentin after root planning and they can not be totally removed with this procedure. So endotoxin could have remained on the root surface after root planning with 20 strokes using curuttes.

Root conditioning with citric acid reduces the inflammatory response. Group 2 specimens have significantly less macrophages, after 7 and 15 days, and significantly less neutrophils, lymphocytes and plasma cells, after 15 days, than group 1 specimens. This results confirm previous findings[30] that citric acid promotes root surface decontamination.

Group 3 specimens also showed less inflammatory cells than the other groups specimens. It is not known any anti-inflammatory action of the PDGF-BB, but Cho et al.[5] found less inflammatory tissue within periodontal defects treated with membranes and PDGF-BB than with membranes alone. An advantage for group 3 specimens is the PDGF-BB activity in the presence of lipopolysaccharide, reversing this toxin inhibitory effects on fibroblasts proliferation[3].

Fibroblasts concentration did not differ significantly between groups. An stimulatory effect of the PDGF-BB over fibroblasts chemotaxis and proliferation could have been masked by the high fibers concentration adjacent to the fragments. The analysis area was always in contact with the fragments, so the high quantity of fibers and low number of cells in this area near to group 3 specimens after 7 days could explain the low fibroblasts concentration.

The ideal growth factor method of administration and concentration for therapeutic use are not known. Some studies uses methylcellulose gel or bovine bone type I collagen as carriers, but their application may raise some potential problems[6]. Animal studies with PDGF-BB used the factor therapeutically at concentrations that varied from 500ng/ml to 500mg/ml[12, 21, 34, 15, 11, 24], but the best results are difficult to be identified because of the high variety of methodologies. The present study showed biologic effects on connective tissue when PDGF-BB is applied without a carrier gel and at a concentration of 100mg/ml, after root conditioning with citric acid.

In summary, the results of this study suggested that PDGF-BB, applied on periodontitis-affected root surfaces that were previously planned and acid conditioned, stimulates connective tissue repair. This effect is more evident within the first week of repair, when the factor promotes collagen fibers deposition. When citric acid conditioning is not performed, inflammatory infiltrate is more intense than when it is performed.

RESUMO

Este estudo avaliou a resposta do tecido conjuntivo à superfície radicular afetada pela doença periodontal após tratamento com PDGF-BB e ácido cítrico, comparando esta terapia com a raspagem radicular e a desmineralização com ácido cítrico. Após a terapia da superfície radicular todas as amostras de raízes foram implantadas em feridas incisionais na região dorsal de ratos e coletadas 3, 7 e 15 dias após a implantação. As análises histológica e morfométrica das amostras implantadas constou de avaliação da densidade de células e avaliação do reparo do tecido conjuntivo adjacente às peças. Concluiu-se que a patogenicidade das amostras radiculares foi reduzida pela desmineralização com ácido cítrico. Os resultados sugerem que o PDGF-BB estimulou o reparo do tecido conjuntivo durante a primeira semana.

Unitermos: Fatores de crescimento; Desmineralização; Cicatrização; Condicionamento radicular; Doença periodontal, regeneração.

Acknowledgments:

The authors are indebted to Tania Mary Cestari and Daniele Santi Ceolin for the techinical assistance.

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